Development of a MMP-responsive Drug Delivery System for De-differentiated Liposarcoma

By

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2020

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A Thesis Submitted in Partial Fulfillment of the Requirements for Graduation with Honors Research Distinction in the Department of Biomedical Engineering at The Ohio State University

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Abstract

De-differentiated liposarcoma (DDLPS) is a malignant soft tissue sarcoma known for its metastatic potential and high rate of local recurrence. Surgical resection remains the standard treatment for DDLPS due to the limited efficacy of systemic chemotherapy and radiation [1]. Chemotherapy is limited by systemic effects that prevent accumulation at the tumor site, thereby requiring higher dosages leading to adverse side effects. We started the development of a targeted drug delivery system that is responsive to specific signals in the tumor and its microenvironment to treat locally recurrent DDLPS. DDLPS is marked by amplification in the chromosomal locus 12q13-15 which is associated with amplifications of the oncoprotein mouse double minute 2 (MDM2) [2]. *Mdm2* is found to be upregulated in nearly all cases of DDLPS and is associated with increased local recurrence rates [3]. Given that *Mdm2* can serve as a reliable diagnostic marker, we wanted to find a downstream product that could be used to prompt controlled drug delivery. Matrix metalloproteinases (MMPs) are a family of endoproteases that contribute towards the degradation of the extracellular matrix (ECM). MMPs have previously been used in targeted drug delivery systems and are upregulated by increased levels of MDM2 [4]. Therefore, the relationship between MDM2 and MMPs demonstrated the potential to be exploited for a controlled drug delivery system. To make this system transferrable to a clinical setting, we began characterization of the MMPs being released from liposarcoma (LPS) cell lines. We used polymerase chain reaction (PCR) to gain a better understanding of how increased *Mdm2* levels impacted MMP expression and found that MMP-1, -2, and -14 mRNA expression was significantly increased in cell lines with higher *Mdm2* expression. Based on these results, we designed four peptide substrates that were amenable to degradation by the identified MMPs. Results indicated a trend in the QGIW and RSLS peptides that suggested preferential degradation by LPS cell lines with high levels of MDM2. To improve the design of these

crosslinkers we used fluorescent peptide zymography to measure which MMPs might be contributing to the degradation of each peptide. We found that MMP-1 and -2 are driving the degradation of the selected substrates by LPS cell lines. Future work will aim to improve the design of the peptide substrates to make them more selective for degradation by MMP-1 and -2 specifically.

Acknowledgements

I would like to take a moment and thank all of those who supported me on this journey and made this accomplishment possible.

First, I want to say my thanks to my research advisor, Dr. Jennifer Leight. You helped me grow so much as a student and a researcher. You always pushed me to think for myself and work hard to gain a deeper understanding of what I was doing and why. You helped me develop my creativity and critical thinking skills which have made me a better researcher.

Second, I want to thank members of the Leight Lab. Abdul, Caitlin, Josh, and Jess you have all been helpful in the advice and wisdom that you have shared. Ameya, you have been my biggest mentor. I appreciate you taking out the time to teach me and help me with my experiments and writing.

Finally, I want to thank my family for advocating for my dreams. Mom and Granny, you both are the reason that I dream big and always strive to be my best. I would not have gotten this far in life without your unwavering love and support.

Table of Contents

List of Tables

List of Figures

Introduction

Motivation

Liposarcoma (LPS) accounts for approximately 20% of all soft tissue sarcomas. LPS develops in any part of the body that contains adipocytes but is most typically found in the limbs or peritoneal cavity [5]. The most common subtypes of LPS are well-differentiated and de-differentiated liposarcoma (DDLPS). Of these, DDLPS presents significant clinical challenges with only a 10% 10-year survival rate due to frequent local recurrence, metastatic potential and deep-seated, unresectable tumors [6].

Current methods for treating DDLPS including surgery, radiation therapy, and chemotherapy remain mostly unchanged since the 1970s. Multiple surgeries are often performed in order to address local recurrence, but with limited improvements in overall patient survival [1]. Doxorubicin, the primary chemotherapy used to treat DDLPS, is classified as anthracycline which means it is a type of chemotherapy that works by intercalating deoxyribonucleic acid (DNA) in cells in order to prevent cell growth and multiplication (Figure 1) [7]. Doxorubicin is delivered systemically, and relies on accumulation of drug at the tumor site for efficacy. By the enhanced permeability and retention (EPR) effect, the drug concentration should be highest in the tumor as a result of hypervascularization and lack of lymphatic draining capabilities (Figure 2) [8]. However, when chemotherapy is given to a patient, it enters systemic circulation and is distributed to the tumor and other tissues based on mass and blood flow rate [9]. With a lack of active tumor targeting, the drug must be delivered in high dosages to have an effect, resulting in significant off-target effects, such as nausea, hair loss and weakening of the cardiac muscle [10]. Additionally, systemic therapies have short half-lives requiring multiple dosages. Moreover,

doxorubicin has an upper-bound of patient responsiveness at only 25% [11]. It is important to note that the ineffectiveness of chemotherapies such as doxorubicin in treating DDLPS tumors is not a result of the type of drug being administered. *In vitro* studies have shown that doxorubicin is effective at killing cells. *In vivo* data, however, shows that systemic therapies struggle to localize treatment to the tumor site. Moreover, drug dosage levels have to be reduced from an adequate concentration needed to treat DDLPS tumors in order to limit systemic toxicity. Because of the drawbacks of current treatments, there is a need for alternatives that can overcome present limitations by restricting delivery of the drug to the tumor site and controlling the release of the drug only when it is needed. A drug delivery system with controlled release properties that can be implanted at the site of the tumor would represent such a solution.

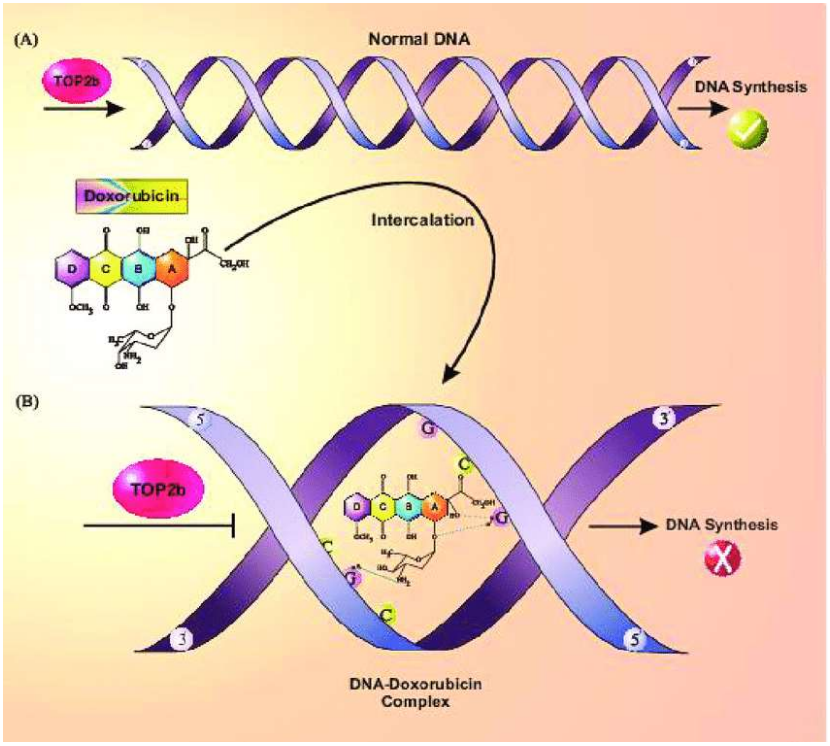


Figure 1: Doxorubicin inhibits DNA synthesis by acting as an DNA intercalating agent [12].

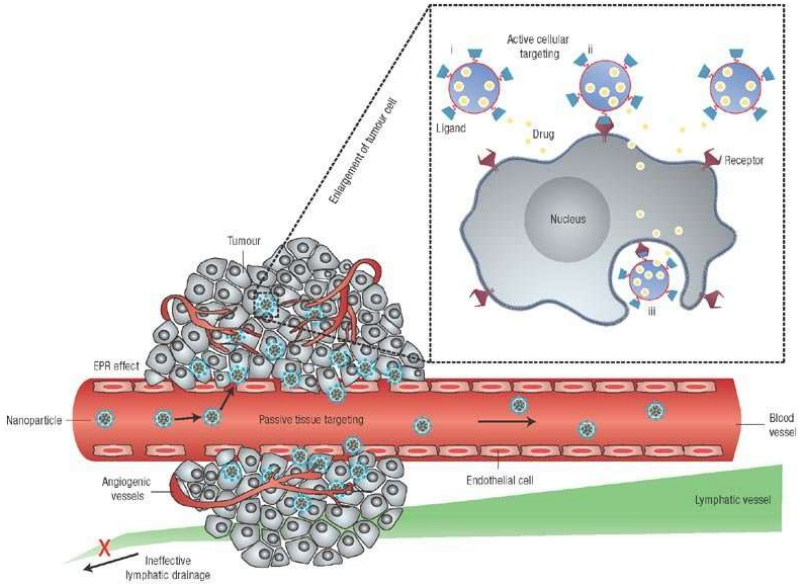


Figure 2: EPR effect results in more distribution of drug to tumor site due to enhanced vascularization and reduced lymphatic drainage [13].

To develop a drug delivery system for DDLPS, properties specific to DDLPS that could prompt the release of drugs needed to be identified. One characteristic of DDLPS is the presence of mouse double minute 2 (MDM2) in nearly 100% of cases. However, MDM2 cannot, itself, prompt release of the drug. MDM2 is not a protease and protease activity has been used extensively in targeted drug delivery systems. Therefore, we needed to identify a downstream regulator of MDM2 that could. DDLPS possesses amplifications in chromosomal locus 12q13-15 which contains *Mdm2*, an oncogene [2]. Data gathered from *in vitro* experiments performed on Simpson-Golabi-Behmel syndrome (SGBS) pre-adipocytes (PAs) showed that MDM2 released in extracellular vesicles (EVs) by DDLPS cell lines, can be transferred to healthy PAs, leading to an increase *Mdm2* copy number and ultimately the activation of MMP-2 (Figure 3) [14]. MMPs contribute towards cancer cell migration and invasion, the initial stages of metastasis, by degrading the extracellular matrix (ECM), supporting angiogenesis, and regulating growth factor bioavailability and activity [15]. Given their proteolytic ability and the fact that

they are upregulated in DDLPS, harnessing the increased MMP activity provides a promising opportunity for a drug delivery system.

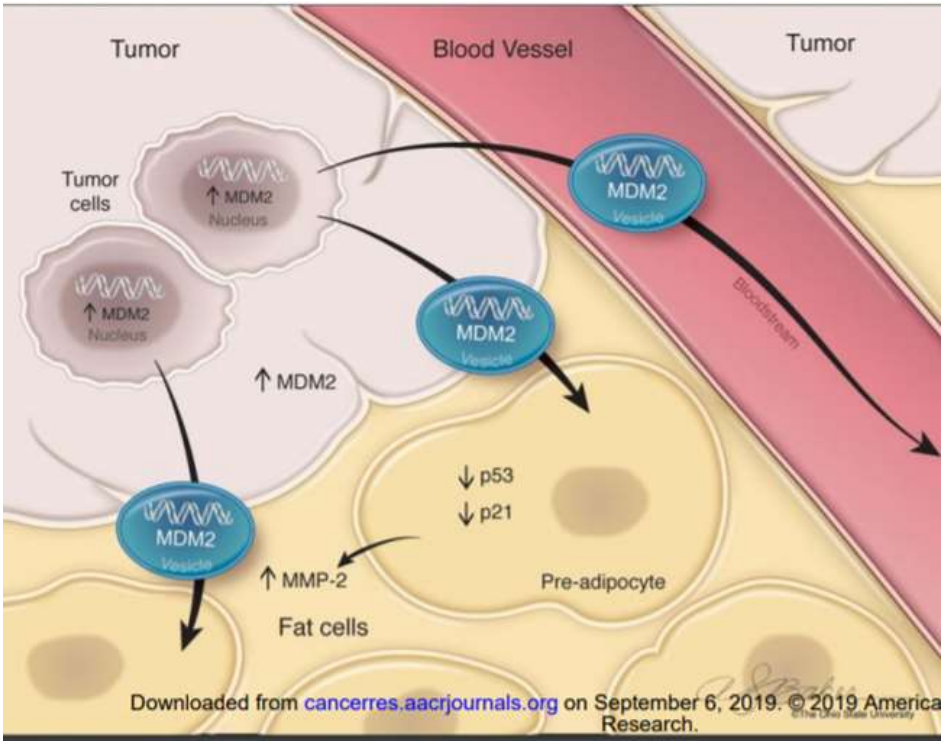


Figure 3: *Mdm2* from LPS cells is transferred to PAs through EVs. The resulting increase in *Mdm2* expression in PAs results in increases in MMP-2 activity [14].

Although several mediums have been explored for the application of controlled drug delivery, hydrogels are particularly appealing for their modular biological and mechanical characteristics, high water content, biocompatibility and low cytotoxicity. There are several ways to control the release of a drug from a hydrogel including mesh size manipulation, network degradation or mechanical deformation. Of these, network degradation through proteolysis of a peptide crosslinker with well-defined degradation represents the most controllable, with half-lives of weeks to months [16]. Given this, we aim to design a system that is responsive to the increased MDM2-dependent MMP activity.

The overall goal of this project is to measure the impact of elevated *Mdm2* copy number on MMP activity *in vitro* and use the information to design an MMP-sensitive controlled drug delivery system. First, we measured the mRNA expression of MMPs typically upregulated in cancer to inform the design of a peptide crosslinker that is amenable to upregulated MMP activity in LPS. Then, we incorporated the selected peptide substrates into a hydrogel assay to measure global MMP activity and preferential degradation by LPS cell lines. Finally, in order to identify which MMPs were responsible for the degradation of the selected peptides, we performed substrate gel zymography. To confirm that the measured degradation was due to *Mdm2*-induced MMP activity, we also treated cells with an MDM2 inhibitor, SAR405838 (SAR). Although we were only able to measure trends between *Mdm2* amplification and peptide degradation, we believe that future work can build upon these results to design a more specific peptide substrate. This treatment method, when paired with surgery, has the potential to reduce damage to healthy tissue and local recurrence rates by primarily targeting tissues with cancerous behavior.

Significance

DDLPS is the most common form of LPS, associated with high local recurrence rates, making it difficult to treat. Radical surgery remains the primary method for treatment as radiation and chemotherapy have shown limited efficacy. There is a need for improved treatment protocols that can cater to its unique characteristics. Our approach involves defining the relationship between MMP activity and elevated *Mdm2* copy numbers measured in nearly all DDLPS cells. With this knowledge, we hope to design a degradable biomaterial that locally delivers

chemotherapeutic drugs to the tumor site. This method could provide a safer and more effective way of treating LPS because it will not rely on accumulation of the drug at the tumor site by the EPR effect. The goal would be to implant the drug delivery hydrogel directly after surgical resection of the tumor, with the drug being released in response to recurrence and subsequent elevated MMP activity. The findings of this project can also impact the development of better treatment protocols for other locally recurrent diseases such as glioblastoma.

Background

MDM2

Cancer development can be attributed to a variety of different factors. However, many types of cancer possess a defective p53 pathway. p53 is a protein whose primary function is to suppress the formation of tumors by repairing defective DNA, arresting cell growth and signaling apoptosis. It is estimated that approximately 50% of all human cancers have some form of p53 mutation [17]. The causes of reduced p53 functionality vary, yet one mechanism is prominent: the negative regulatory feedback loop between p53 and MDM2. Many types of cancers including LPS possess overamplification of *Mdm2*. As an oncoprotein, it is known to target tumor suppressors such as p53. The primary functions of p53 such as regulating the cell cycle and triggering apoptosis, are disrupted by the binding of MDM2. Acting as an E3 ubiquitin ligase, MDM2 monomerically ubiquitinates p53, marking it to be degraded by proteasomes (Figure 4) [18]. MDM2 is upregulated in nearly 100% of DDLPS cases [19]. It has therefore been used as a reliable diagnostic marker of LPS and has been labeled as an oncogene that promotes tumor formation.

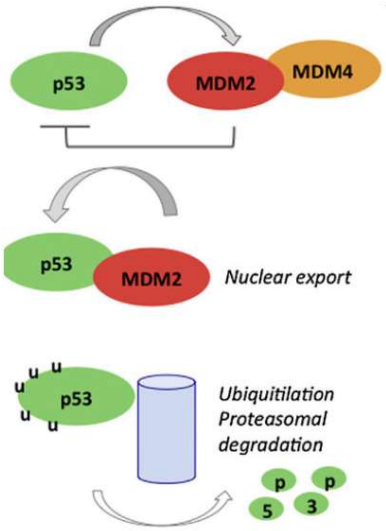


Figure 4: MDM2 marks p53 for degradation by the proteasome. DDLPS is marked by overamplification of the oncogene, *Mdm2*. MDM2 binds to the cell-cycle regulator p53 and marks it for degradation in the proteasome. Degradation of p53 leads to tumor progression [20].

MMPs

MMPs are a family of zinc-dependent endopeptidases that have been identified as critical regulators of cancer progression. The primary function of MMPs involves the degradation and remodeling of the ECM. There are currently over 20 identified MMPs in the human body and they have a role in cleaving a variety of substrates such as chemokine, growth factors, cell-binding proteins, and collagen [21]. MMPs are initially translated as inactive, pro-MMPs. Upon activation they become proteolytically active and contribute to cellular invasion by cleaving stromal macromolecules. This is important because by allowing cells to invade, they can drive primary tumor growth, local recurrence and metastasis. When studying MMPs, expression levels can be measured using polymerase chain reaction (PCR) which accounts for MMPs at the transcriptional level or western blot analysis which accounts for MMPs at the protein level. Activity levels, on the other hand, are measured using peptide zymography. It is critical to study MMPs on both levels because expression levels do not have a direct correlation with activity levels. Clinically, researchers have established a positive correlation between upregulation in MMP activity and a poor patient prognosis. For example, in a study of patients with human breast cancer, a high expression of MMP-2 and MMP-9 was found in 83.75% of tissue samples. They concluded there was a closely linked relationship between lymph node metastasis and tumor staging [15]. MMP-targeting inhibitors have been tested clinically but have not been highly successful, likely due to compensation strategies and off-target effects. Gaining a better understanding of how these enzymes are regulated in cancer is critical. It can lead to the development of more efficacious treatment strategies that use MMPs as an asset (such as the controlled drug delivery system we are designing) rather than a target.

SAR

SAR is a small molecule that inhibits the interaction between MDM2 and p53. Inhibition of the interaction between MDM2 and p53 occurs when SAR binds to MDM2 and is correlated with decreased MMP activity [14]. SAR binds to MDM2 with a K(i) = 0.88 nmol/L, demonstrating a high affinity that is greater than 10, 50, and 1000 times more potent than other MDM2 inhibitors: MI-219, nutlin-3a, and the p53 peptide, respectively. Uniquely, SAR possesses the same important p53 amino acid residues that MDM2 binds to with high specificity. This specificity is bolstered by supplemental interactions of SAR with the N-terminal region (region that binds to p53) of MDM2. Studies have shown that SAR also has the ability to activate wild-type p53. Results showed tumor regression and growth inhibition in mouse xenograft models of SJSA-1 osteosarcoma, RS4;11 acute leukemia, LNCaP prostate cancer, and HCT-116 colon cancer [22]. This makes SAR an ideal candidate for comparing the effects of decreased MDM2 interaction with p53 on MMP activation and preferential degradation of a peptide substrate within LPS cell lines. We hypothesized that by disrupting the interaction between MDM2 and p53 using SAR, MMP activity would decrease.

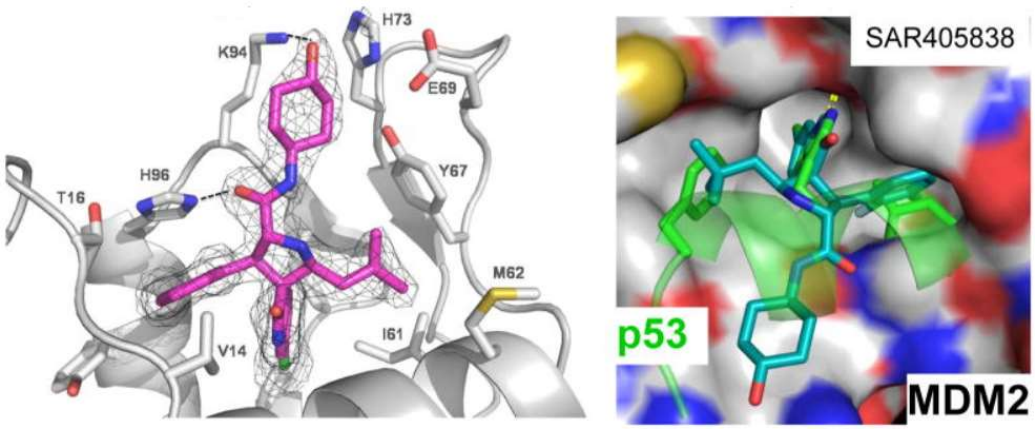


Figure 5: Co-crystal Structure of SAR405838/MDM2 at 2.1 Å resolution (right) Superposition of SAR405838/MDM2 and p53 peptide/MDM2 co-crystal structure (left) [22].

Controlled Drug Delivery

Controlled drug delivery systems are a technology that have been developed over the past 60 years. The first generation (1950-1980) concentrated on the development of oral and transdermal controlled release formulations. The second generation (1980-2010) concentrated on the development of zero-order release systems, self-regulated drug delivery systems, long-term depot formulations, and nanotechnology-based delivery systems. Now, in the third generation the focus is on overcoming the limitations of previous generations and advancing the compatibility of these systems in clinical applications and *in vivo* [23]. Given the current focus, the purpose of controlled drug delivery systems has been established as maximizing the efficacy of therapeutic drugs while minimizing the adverse effects by releasing only the necessary dose at the appropriate time. This technology is needed to advance current treatment methods with numerous side effects and minimal success in cancers such as DDLPS. Examples of current drug delivery systems include carbon nanotubes (CNTs), micelles and hydrogels. CNTs are artificial one-dimensional nanomaterials that are composed of carbon and sheets of graphene rings that were rolled to create the hollowed-out nanotube shape seen in Figure 6. When loaded with doxorubicin, CNTs are very effective at treating multidrug resistance in leukemia cells. They have not yet been shown to be an effective treatment for soft tissue sarcomas such as DDLPS. Micelles are spherical nanomaterials that are composed of amphipathic molecules that spontaneously form a hydrophobic interior and hydrophilic exterior in aqueous solutions (Figure 6). Micelles are useful for reducing systemic toxicity and improving delivery of drug to the original tumor site in breast, lung and ovarian cancers [24]. One of the major drawbacks of micelles, however, is their instability, especially in the presence of environmental changes. They have a tendency to dissociate prior to reaching their destination [25]. Hydrogels are a three-

dimensional network of hydrophilic polymer chains held together by crosslinkers. They are very versatile in terms of their applications such as forming material for artificial organs and contact lenses, biosensors and drug delivery vehicles. Hydrogels are advantageous because of their biocompatibility, minimal cytotoxicity, high biomacromolecule encapsulation efficiency and ability for drug delivery systems to be administered in various forms (i.e. oral, rectal, ocular, epidermal, and subcutaneous). Hydrogels also offer multiple mechanisms for controlled drug release such as mesh size manipulation, network degradation or mechanical deformation. For these reasons and the sensitivity to MMP degradation, we chose hydrogels as our form of drug delivery system.

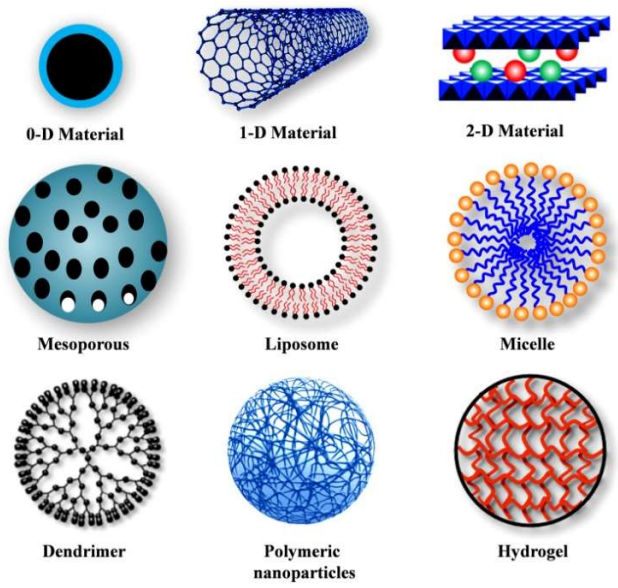


Figure 6: Drug delivery system nanocarriers. Drugs are conjugated to the selected nanomaterials and are distributed in a targeted and controlled manner to the tumor site [24].

MMPs in Controlled Drug Delivery Systems

Given the strong positive correlation with MDM2, MMPs demonstrated the potential to be incorporated in our controlled drug delivery system. Also, MMPs have been used previously in targeted drug delivery systems. As stated in my section on MMPs, upregulation in MMP activity is associated with cancer. This has established MMPs as predictable cancer biomarkers. Therefore, when MMP activity has been tailored to targeted drug delivery systems, they have been described as a strong and stable stimulus for specific tumor targeting [26]. In fact, studies have shown MMPs can help prompt cellular endocytosis and heightened penetration into deep-seated tumors [27]. Figures 7 and 8 show some examples of the plethora of modalities that MMPs are used in for targeted drug delivery. The essential mechanism remains the same whereby an MMP degradable substrate is incorporated into the drug nanocarrier. The upregulated MMP activity that cancer cells yield would result in increased degradation of this substrate and the drug would then be released into the tumor microenvironment. For our system in particular, MMP degradable peptide substrates used as crosslinkers are conjugated into hydrogels. MMPs then degrade these cross linkers in order to release the drug.

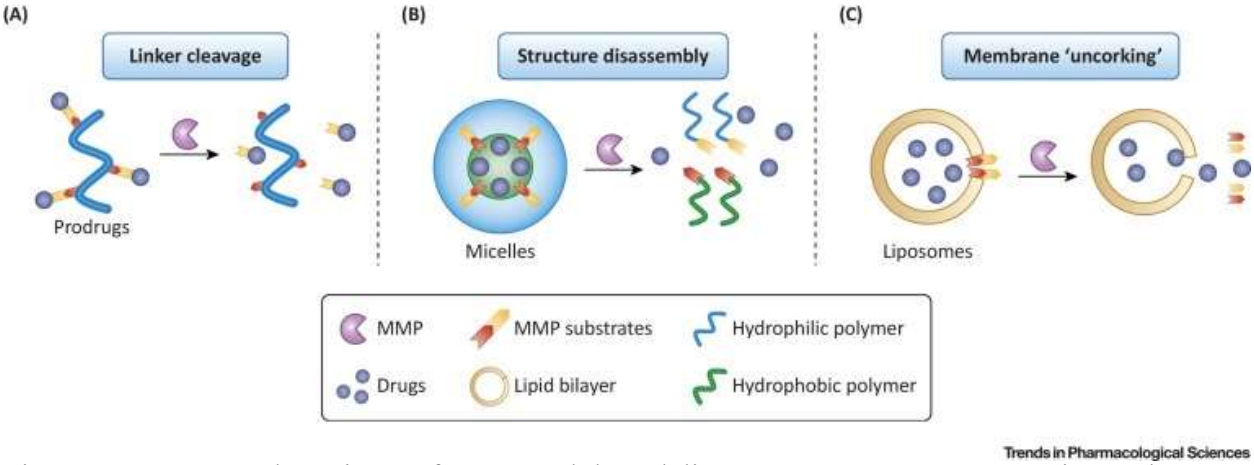


Figure 7: MMPs used as triggers for targeted drug delivery systems. Drugs are conjugated to nanocarriers with MMP degradable substrate. MMPs degrade substrate and prompt release of drug [28].

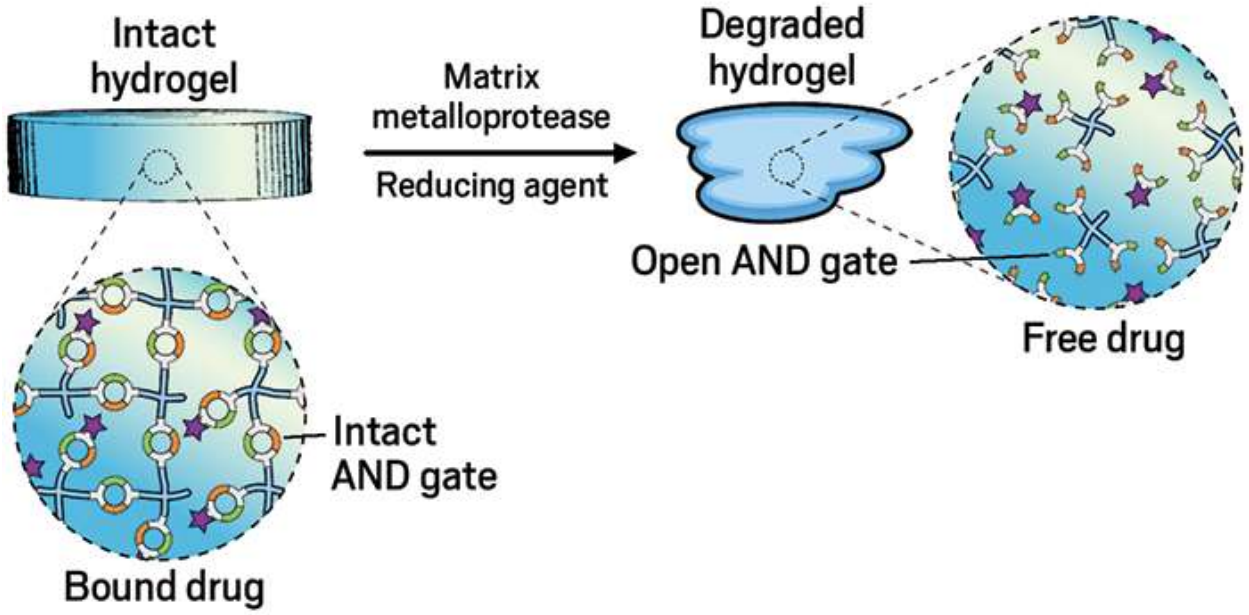


Figure 8: MMPs act as reducing agent in hydrogel-based controlled drug delivery systems. Drugs are conjugated to hydrogel with MMP degradable peptide substrate. MMPs degrade substrate and prompt release of drug [29].

Materials and Methods

Cell Culture

Lisa-2, Lipo863, and Lipo246 LPS cell lines were provided by the Pollock Lab. Lisa-2, Lipo863, and Lipo246 presented with low, medium and high *Mdm2* amplification, respectively [30]. They were cultured in Dulbecco's Modified Eagle Medium (DMEM) media. The media contained 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine. The cells were maintained in a humidified incubator at 37ºC and 5% CO_2.

Peptide Synthesis

Peptides GGPQG↓IWGQK^{Dde}(Adoo)C (abbreviated as QGIW throughout the text and figures to denote the P2-P2' residues; ↓ indicates cleavage site); GPLA↓C(MeOBzl)WARK^{Dde}DDK(Adoo)C (abbreviated as LACW throughout the text and figures to denote the P2-P2' residues; ↓ indicates cleavage site); K^{Mtt}GPRS↓LSGK^{Dde}(Adoo)C (abbreviated as RSLS throughout the text and figures to denote P2-P2' residues; ↓ indicates cleavage site); GPL↓GL(Dap)^{Dde}AR(Adoo)C (abbreviated as PLGL throughout the text and figures to denote P2-P2' residues; ↓ indicates cleavage site) were synthesized using Fmoc solid phase peptide synthesis (CEM Liberty Blue Peptide Synthesizer, Matthews, NC) using a Rink Amide MBHA resin (EMD-Millipore, Burlington, MA). The QGIW peptide is a commonly used, collagen I-derived sequence [31]. LACW is an artificial, commercially available peptide that has been optimized for MMP-14 detection [32]. RSLS was designed and implemented for the detection of MMP-2 and MMP-9 [33]. PLGL is a commonly used, broad-spectrum MMP-sensing peptide that has been characterized for the detection of MMP-1, -2, -7, -8, -9, -12, -13, -

14, -15, -16 [34]. All Fmoc protected amino acids and Fmoc-8-amino-3,6-dioxaoctanoic acid (Adoo) were purchased from Chem-Impex (Chem-Impex, Wood Dale, IL).

Peptides were functionalized with a quencher (dabcyl) and fluorophore (fluorescein) [35]. Briefly, a dabcyl succinimidyl ester (Anaspec, Fremont, CA) was coupled to the amino terminus of the peptide in dimethyl formimide (DMF) with 6 eq. N,N'-diisopropylethylamine (DIPEA) and reacted overnight. For the RSLS peptide, the dabcyl succinimidyl ester was coupled to the side-chain amine in the N-terminal K^{Mtt}. K^{Mtt} was deprotected using 9 washes of 1.8% TFA in DCM for three minutes each. An orthogonally protected lysine (K^{Dde}) or diaminopropoinoic acid (Dap^{Dde}) was then deprotected twice in 2% hydrazine monohydrate in DMF for 10 min and a fluorescein NHS ester (Anaspec) was coupled to the resultant free amine in the same manner as dabcyl. The peptides were cleaved from the resin beads by incubating in a cleavage cocktail of trifluoroacetic acid, phenol, triisopropylsilane and water (95/2.5/1.25/1.25 v/v) for 3 h at room temperature, and precipitated in chilled diethyl ether 3 times. Peptides were purified by reverse phase high performance liquid chromatography (RP-HPLC; Hitachi, Schaumburg, IL) (QGIW: 30-70%; LACW: 52-70%; RSLS: 30-70%; PLGL: 10-70% gradient of acetonitrile at 2% ACN/min) and molecular weight (QGIW: 1884 Da; LACW: 2336 Da; RSLS:1743 Da; PLGL:1481 Da) was verified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF; Bruker, Billerica, MA).

Real Time-Polymerase Chain Reaction

Cells were plated at 30,000 cells/cm^2 and cultured for 24 h in complete growth medium. RNA was isolated using the RNeasy RNA isolation kit (Qiagen) according to the manufacturer's protocol. 2 ng of mRNA was then reverse transcribed using the iScript reverse transcription kit (Bio-rad), and used for qRT-PCR. Primers were obtained from the Harvard Primer Bank (https://pga.mgh.harvard.edu/primerbank/) and verified using Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). qRT-PCR was run using SYBR Green (Thermo Fisher Scientific) for detection. Primers were used at 900 nM, and expression levels were normalized to the endogenous control, 18S (Thermo Fisher Scientific). Primer sequences were as follows:

MMP-1	F: AAGATGAAAGGTGGACCAACAATT R: CCAAGAGAATGGCCGAGTTC
MMP-2	F: AACTACGATGACGACCGCAAGT R: AGGTGTAAATGGGTGCCATCA
MMP-13	F: CATGAGTTCGGCCACTCCTT R: CCTCGGAGACTGGTAATGGC
MMP-14	F: AAGGCCAATGTTCGAAGGAA R: GGCCTCGTATGTGGCATACTC

Table 1: Primer Sequences

Hydrogel Encapsulation

Hydrogel composition consisted of 10 wt% 8-arm 40K Poly(ethylene glycol) norbornene (PEG-NB), 12.75 mM MMP-degradable crosslinker (0.7 thiol:ene), 0.5 M NaOH, 1 mM RGD pendant peptide, 2 mM Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), and 0.25 mM MMP peptide. The MMP-degradable crosslinker is flanked by thiol moieties that are able to conjugate with the norbornene groups in the PEG. RGD is a cell adhesion peptide sequence for cells to adhere to it. LAP is a photoinitiator that catalyzes the thiol-ene reaction in the presence of ultraviolet (UV) light (365 nm). Various MMP degradable peptides were used including RSLS, PLGL, QGIW, and LACW in order to identify which substrate would be best suited for preferential degradation by MMPs upregulated by *Mdm2* overamplification. A concentrated solution of cells and phosphate buffered saline (PBS) were added to the hydrogel mixture. Triplicates of each condition were transferred to a black, round-bottom 96-well plate. Plate was placed under UV light (4 mW/cm^2; 365 nm) for 3 minutes. 150 µL of DMEM media +/- SAR treatment at a concentration of 0.3 µM was the added to the respective wells. Cells were transferred to an incubator at 37ºC and 5% CO_2. After 18 h, 15 µL of AlamarBlue at a concentration of 10% was added to each well. At 24 h, fluorescence readings were captured using a SpectraMax M5 plate reader at 494 nm excitation/521 nm emission wavelength for peptide degradation and 560 nm excitation/590 nm emission for alamarBlue. Averages of each 3 x 3 well scan were calculated for the detection of overall peptide degradation.

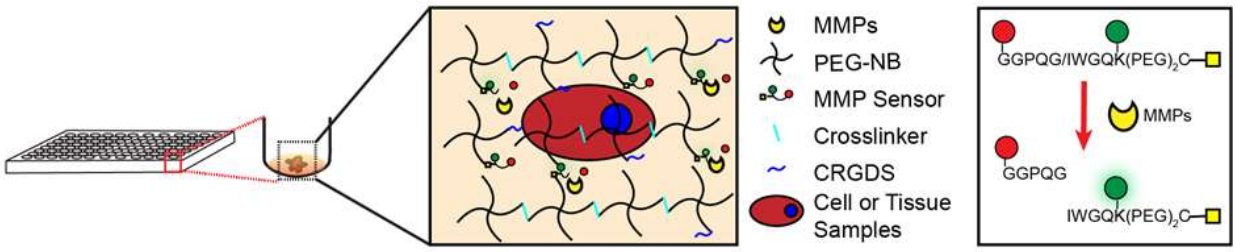


Figure 9: Schematic of the PEG hydrogel platform functionalized with the fluorescent peptide biosensor. Degradation of the biosensor by cell-secreted MMPs results in separation of the quencher and fluorophore resulting in an increase in fluorescence intensity.

Peptide Zymography

For zymographic analyses, cells were seeded at a density of 30,000 cells/cm^2 in duplicate wells of a 6-well plate for 24 h in 10% FBS media, washed with PBS twice and then cultured in serum-free media for an additional 24 h. Media samples were collected and concentrated using 10-kDa Amicon Ultra Centrifugal Filter Units (EMD-Millipore). Conditioned cell media samples were loaded under non-reducing conditions onto prepared fluorescent peptide zymography gels. Fluorescent peptide zymography gels were prepared as previously described [36]. The samples were electrophoresed at 120 V at 4ºC for 1.5 h. Following electrophoresis, gels were washed three times for 15 min each at room temperature under gentle agitation in renaturing buffer containing 2.5% Triton X-100 in 50 mM Tris-HCl, pH 7.5. Gels were then transferred to a developing buffer solution, 1 µM $ZnCl_2$ and 5 mM $CaCl_2$ in 50 mM Tris-HCl, pH 7.5, overnight at 37ºC under gentle agitation. Fluorescent images of the fluorescent peptide gels were captured at 24 h using a GE Amersham Typhoon 9410 Gel Imager (Excitation 488/Emission 521).

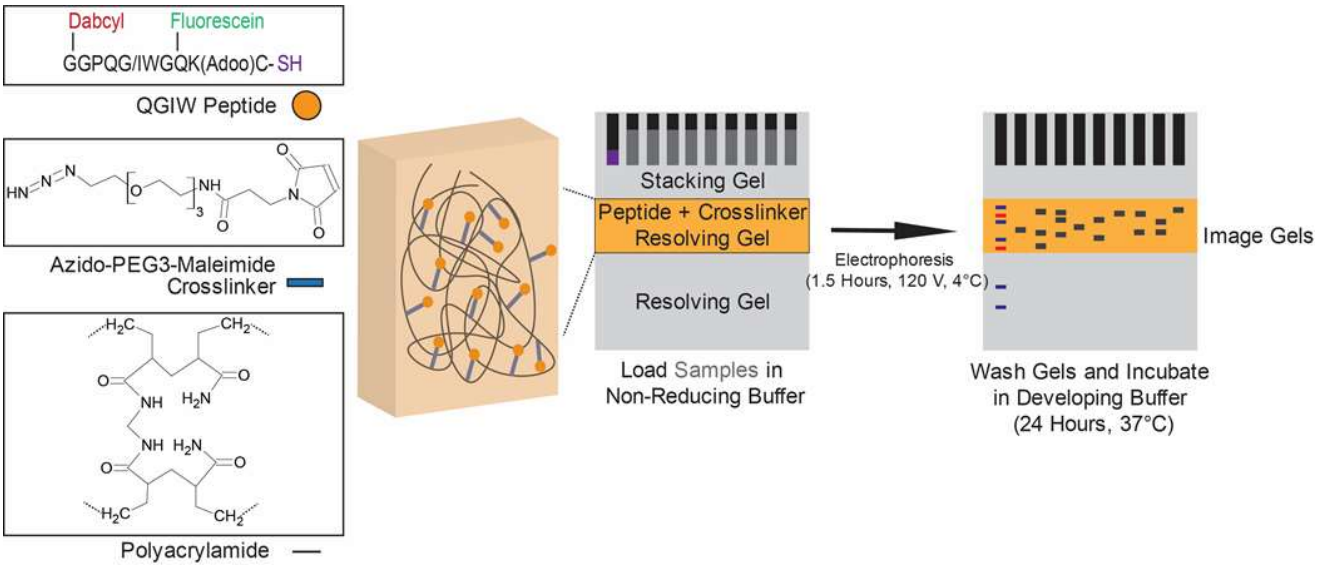


Figure 10: Schematic of fluorescent peptide zymography. Fluorescent peptides incorporated into polyacrylamide gels using a crosslinker. Gels are electrophoresed and degradation can be visualized and quantified based on change in fluorescent intensity [36].

Statistical Analysis

For qt-PCR, data analysis was performed by two-way ANOVA followed by Dunnett's multiple comparisons test using the Lisa-2 cell line as the control.

For hydrogel encapsulation data analysis, triplicate wells were averaged and the background values of gels with no cells were subtracted from cell-containing gels for each of the four peptides screened. To identify a peptide that is preferentially degraded by LPS cells with overamplification of *Mdm2*, RFU values of LPS cell lines at 24 h were normalized to Lisa-2 cells. Analysis was performed by one-way ANOVA with Dunnett's multiple comparisons test using the Lisa-2 cell line as the control.

For peptide zymography data analysis, a one-way ANOVA followed by Tukey's multiple comparisons test was performed. Graphs were created and statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Sofware, Inc, San Diego, CA). At least three independent experiments were carried out for each experiment and data are shown as mean±SD (n=3), unless otherwise noted.

Results and Discussion

Controlled Drug Delivery System Peptide Screen

The purpose of the PCR experiments was to investigate MMP mRNA expression. We wanted to understand how the suppression of MDM2 would change MMP expression levels. This would give us an idea of the specific MMPs we could target for the development of a controlled drug delivery system. Lisa-2 cells have no amplification of *Mdm2.* Lipo863 and Lipo246 cell lines have low and high levels of *Mdm2* amplification, respectively. The MMPs selected for PCR experimentation were chosen based on their history of being upregulated in cancer and the desire to have a range of MMP types. MMP-1 and MMP-13 are collagenases, MMP-2 is a gelatinase and MMP-14 is membrane-bound and a primary degrader of collagen.

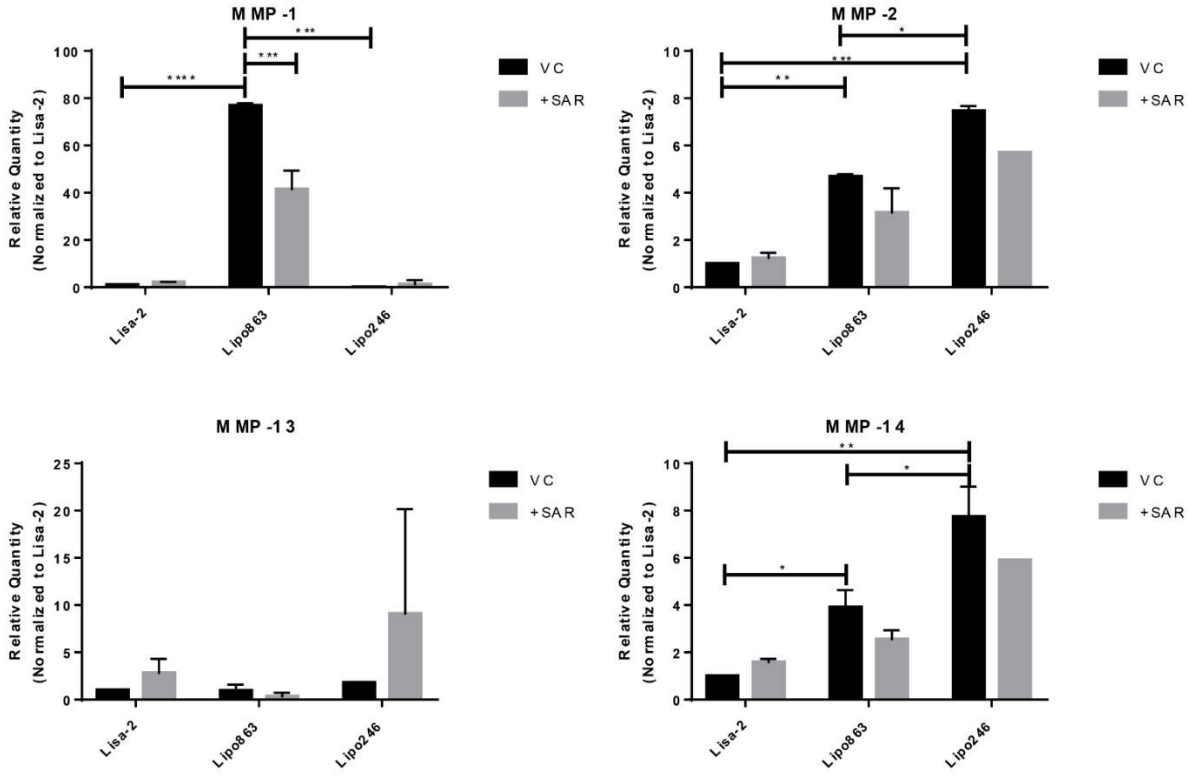


Figure 11: MMP-1, -2, -13, -14 mRNA expression in LPS cell lines. VC: vehicle control (DMSO). Results are represented as n=4, mean±SD. *p<0.05, **p<0.01, ***p<0.005.

MMP-1 expression was upregulated approximately 80-fold in Lipo863 cells compared to Lisa-2 cells (Figure 11). Treatment of Lipo863 cell lines with the MDM2 inhibitor, SAR, resulted in a decrease in MMP-1 expression by 50% (approximately 40-fold higher compared to Lisa-2). MMP-2 expression was upregulated in the Lipo863 (5-fold) and Lipo246 (7-fold) cell lines. SAR treatment decreased MMP-2 expression by ~25% in both cell lines. A similar trend was measured in MMP-14. Interestingly, SAR treatment of Lisa-2 cell lines appeared to moderately increase MMP expression in all cases. This is likely due to the fact that Lisa-2 cell lines do not exhibit any amplification of *Mdm2*. Previous work has shown that treatment of Lisa-2 cell lines with SAR, while increasing p53 expression levels, also has a positive regulatory feedback effect that results in increased *Mdm2* expression [37]. This may in turn have a downstream effect that increases MMP expression, and ultimately activity. Lipo863 and Lipo246 cells, on the other hand, have increased copy numbers and overamplification of *Mdm2*. Normally, these cells have little or no functioning p53 protein. P53 has been previously identified as a negative transcriptional regulator of MMP expression [37]. Therefore, increased p53 expression from SAR treatment can lead to decreased MMP transcription. This indicates two potential effects of SAR treatment on MMP expression. The responses toward the SAR treatment are dependent on whether cells have normal p53 and *Mdm2* levels or low p53 and high *Mdm2* levels. There is a trend showing increased upregulation of various MMP expression levels with increased *Mdm2* amplification. This demonstrated an association between MMP expression and *Mdm2* amplification in DDLPS. This is indicative of a potential need to develop different controlled drug delivery systems targeting different MMPs to cater to the varying expression levels of different cell lines.

Assessment of Peptide Degradation

The purpose of the hydrogel encapsulation experiments was to study MMP peptide degradation. We wanted to determine if there was a specific peptide that was preferentially degraded by MMPs secreted by DDLPS cancer cell lines with amplification of *Mdm2* compared to those with no or low amplification of *Mdm2*. The peptides selected were chosen based on preferential degradation by the MMPs studied in the PCR experiments. PLGL serves as a general MMP sensor peptide. LACW is preferentially degraded by MMP-14 [38]. QGIW is preferentially degraded by the collagenases such as MMP-1, MMP-8, and MMP-13 [39]. RSLS is preferentially degraded by the gelatinases, MMP-2 and MMP-9 [40].

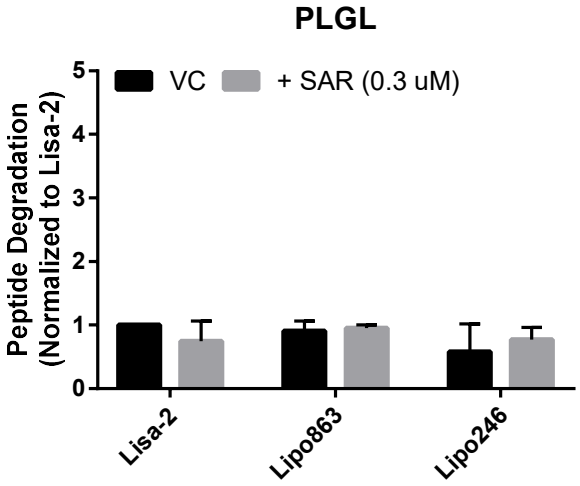


Figure 12: PLGL Peptide Degradation
Results are represented as n=4, mean±SD. VC: vehicle control (DMSO).

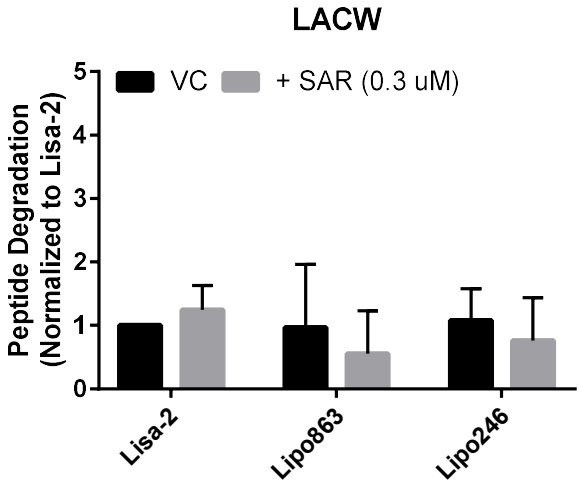


Figure 13: LACW Peptide Degradation
Results are represented as n=4, mean±SD. VC: vehicle control (DMSO).

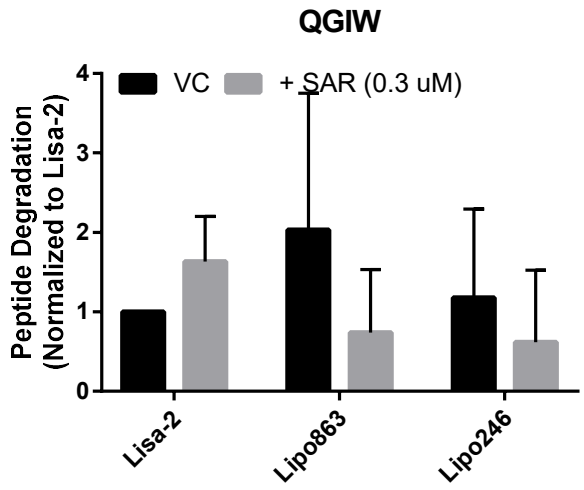


Figure 14: QGIW Peptide Degradation
Results are represented as n=4, mean±SD.
VC: vehicle control (DMSO).

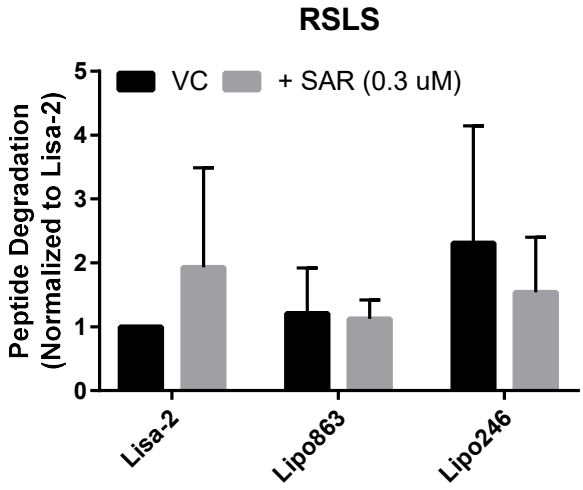


Figure 15: RSLS Peptide Degradation
Results are represented as n=4, mean±SD.
VC: vehicle control (DMSO).

We want a peptide substrate whose degradation increases from Lisa-2 to Lipo863 to Lipo246 with *Mdm2* levels. Figure 12 shows that the PLGL peptide does not display a trend in increased degradation with *Mdm2* overamplification. These results indicate PLGL is not an ideal candidate for our controlled drug delivery system. Additionally, the SAR treatment was used to investigate whether peptide degradation was the result of MDM2-induced MMP activity. We hypothesized that SAR treatment would inhibit MDM2 and ultimately decrease MMP activity. This was only the case for the Lisa-2 cells. The cell lines that should have been the most impacted by SAR inhibition, Lipo863 or Lipo246, slightly increased degradation after treatment.

Figure 13 shows that the LACW peptide does not display a trend in increased degradation with increased *Mdm2* overamplification status. These results indicate LACW is not an ideal candidate

for our controlled drug delivery system. Figure 13 demonstrates a trend with increasing peptide degradation from Lipo863 to Lisa-2 to Lipo246 cells. Upon SAR treatment, peptide degradation increased for Lisa-2 cells and decreased for Lipo863 and Lipo246 cells. This follows a similar trend seen in the PCR results. Although the degradation of the LACW peptide may be controlled by MMP secreted LPS cell lines, it does not appear to be correlated with *Mdm2* levels.

Figure 14 shows that the QGIW peptide does display a trend in increased degradation with increased *Mdm2* amplification. Lisa-2 cells, those with no *Mdm2* amplification had the lowest peptide degradation. Though the expectation was that the highest peptide degradation would be by the cell line with the highest *Mdm2* amplification (Lipo246), this was not the case. The Lipo863 cells exhibited a much higher level of peptide degradation. Though this does not follow the exact degradation trend we expected, with adjustments to the peptide sequence, the peptide could become more favorable to Lipo246 peptide degradation. Upon SAR treatment, peptide degradation increased for Lisa-2 cells and decreased for Lipo863 and Lipo246 cells, as expected based on the PCR data.

Figure 15 shows that the RSLS peptide does display a trend in increased degradation with increased *Mdm2* amplification status. It follows the trend we expected to see where the cells with no overamplification of *Mdm2*, Lisa-2, have the lowest peptide degradation and the cells with the highest overamplification of *Mdm2*, Lipo246 have the highest peptide degradation. These results showing evident preferential degradation with *Mdm2* amplification status indicate RSLS as the optimal peptide substrate to be incorporated into the controlled drug delivery system. However,

the differences in degradation between the cell lines were not statistically significant. SAR treatment increased peptide degradation by Lisa-2 cells and decreased peptide degradation by Lipo863 and Lipo246 cells, as seen previously.

The peptide degradation results correspond with the results seen in the PCR experiments. They exhibit a trend showing that the impact of SAR treatment is based on p53 and *Mdm2* status within cells. In cell lines with no overamplification of *Mdm2*, such as the Lisa-2 cells, SAR treatment has been shown to not have an effect on p53 levels [37]. The SAR treatment has been shown, however, to have an effect on MDM2 whereby levels are increased in cell lines with no overamplification [37]. Therefore, a potential downstream effect of increased MDM2 expression could be an increase in MMP expression. The increase in MMP expression levels with SAR treatment seen in the Lisa-2 cells in the PCR experiments likely prompted an increase in MMP proteolytic activity and ultimately increased peptide degradation. The decreased MMP expression levels with SAR treatment seen in the Lipo863 and Lipo246 cells resulted in the opposite outcome: decreased MMP proteolytic activity and ultimately decreased peptide degradation. Although the peptide screen did not reveal a definitive difference in peptide degradation with increased MDM2 expression (or a statistically significant decrease upon SAR treatment), the QGIW and RSLS peptide do appear to show a trend in that direction. By understanding the secreted MMPs that are likely driving the degradation of these peptide substrates, we can further improve the selectivity of the drug delivery platform.

Evaluation of Proteolytic Activity Causing Peptide Degradation

The purpose of the peptide zymography experiments was to investigate what specific MMPs were contributing to the degradation of the various peptides. Determining which MMPs are actually cleaving the peptides can help indicate how the peptide designs can be refined for improved preferential degradation in future peptide substrates.

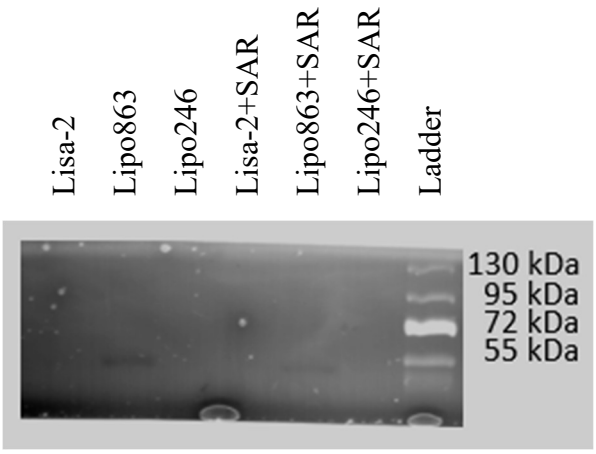


Figure 16: Fluorescent peptide zymography of the PLGL peptide.

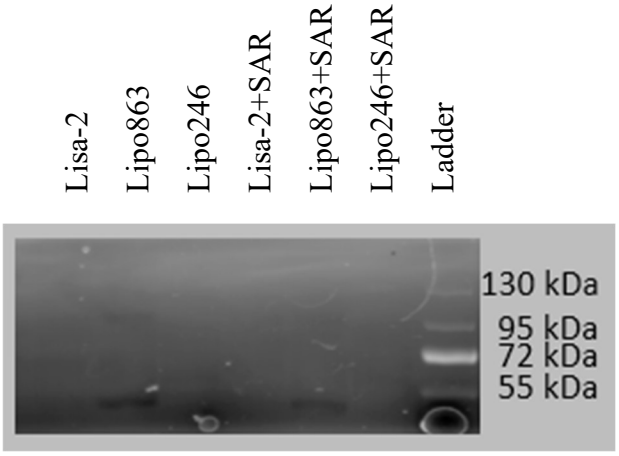


Figure 17: Fluorescent peptide zymography of the LACW peptide.

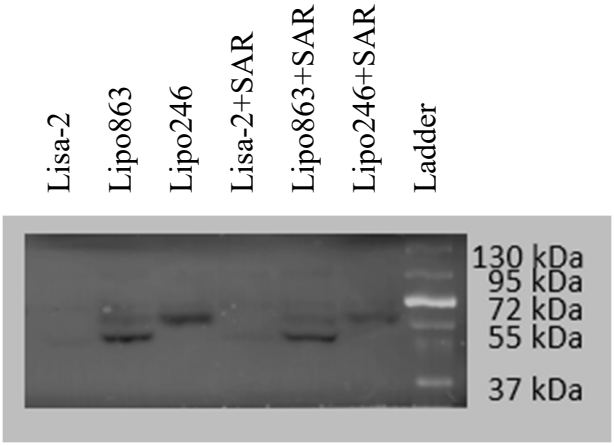


Figure 18: Fluorescent peptide zymography of the QGIW peptide.

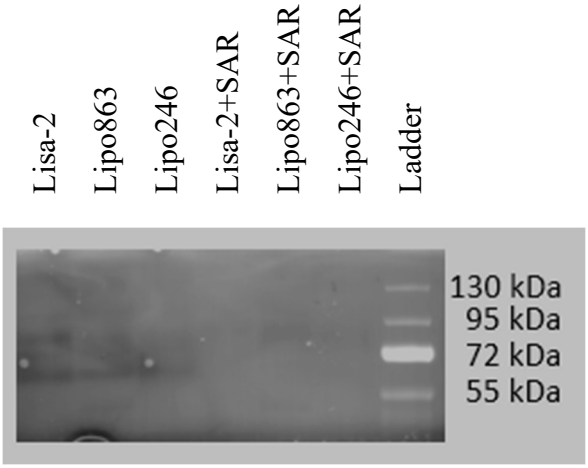
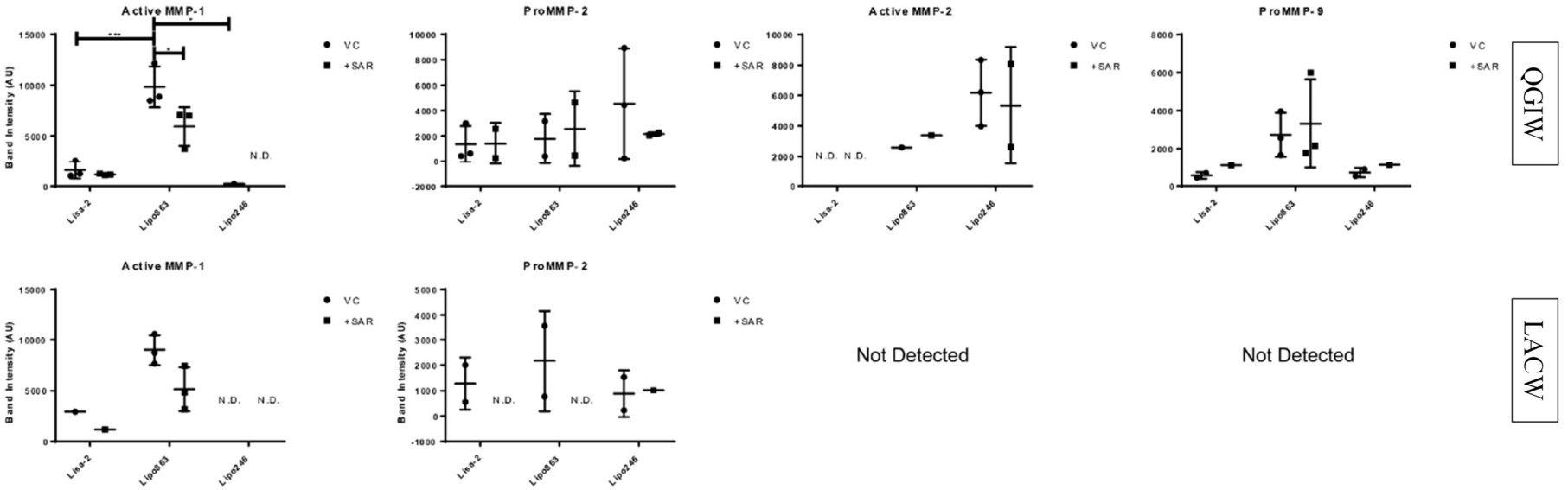
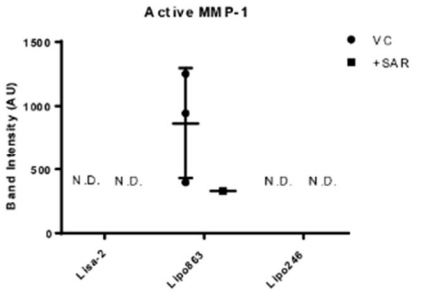


Figure 19: Fluorescent peptide zymography of the RSLS peptide.


Active MMP-1
Band Intensity (AU)
15000
10000
5000
0
***
*
*
N.D.
Lisa-2
Lipo8.63
Lipo2.46
VC
+SAR
ProMMP-2
10000
8000
6000
4000
2000
0
-2000
Active MMP-2
N.D. N.D.
ProMMP-9
8000
6000
4000
2000
0
QGIW
Active MMP-1
N.D. N.D.
ProMMP-2
5000
4000
3000
2000
1000
0
-1000
N.D. N.D.
Not Detected
Not Detected
LACW

RSLS



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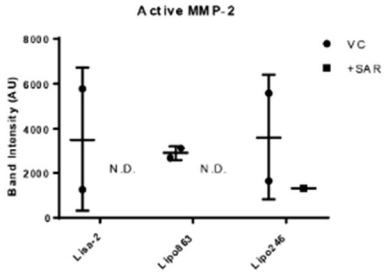
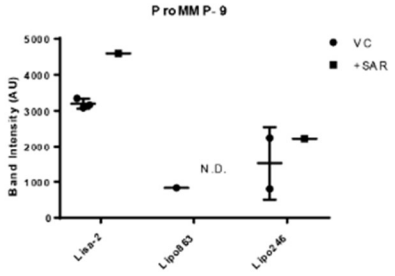
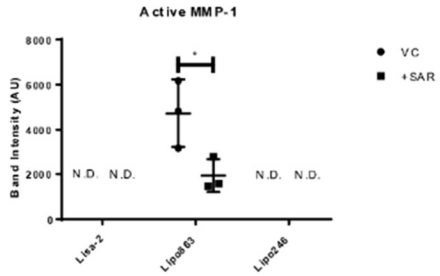




PLGL



Not Detected

Not Detected

Not Detected

Figure 20: Fluorescent peptide zymography quantification. Conditioned media from Lisa-2, Lipo863, and Lipo246 cell lines was collected after culturing in serum free media +/- SAR (0.3 μM) for 24 h. The conditioned media samples were electrophoresed in peptide zymography gels containing the QGIW, LACW, RSLS, and PLGL fluorescent peptide substrates. Results are represented as n=3, mean±SD. *$p<0.05$, **$p<0.01$, ***$p<0.005$.

Figure 16 of the PLGL peptide shows cleavage by active MMP-1 (molecular weight = 54 kDa) by Lipo863 with and without SAR. Upon SAR treatment, MMP activity levels decreased as shown by the decrease in fluorescence intensity by 41% ($p<0.05$) in Figure 20. Despite the fact that a significant difference in activity was observed, it was only in the Lipo863 cell line. Additionally, net degradation of the PLGL peptide by each cell line revealed no increase in degradation by cell lines with higher levels of *Mdm2*.

Figure 17 of the LACW peptide shows cleavage by active MMP-1 by Lisa-2 and Lipo863 cells with and without SAR. It also shows cleavage by pro MMP-2 (molecular weight = 72 kDa) by Lisa-2, Lipo863, and Lipo246 cells without SAR and Lipo246 cells with SAR. Finally, it shows cleavage by active MMP-2 (molecular weight = 62 kDa) by Lipo246 cells without SAR treatment. Upon SAR treatment, MMP-1 activity levels in the Lisa-2 cells decreased as shown by the decrease in fluorescence intensity by 40% in Figure 20. Upon SAR treatment, MMP-1 activity levels in the Lipo863 cells decreased as shown by the decrease in fluorescence intensity by 57% in Figure 20. Upon SAR treatment, pro MMP-2 levels in the Lisa-2 and Lipo863 cells were undetectable. Upon SAR treatment, pro MMP-2 levels in the Lipo246 cells increased as shown by the increase in fluorescence intensity by 15% in Figure 20. For active MMP-1, Lipo863 cells exhibited more activity than Lisa-2 cells. For pro MMP-2, Lipo863 cells exhibited the most MMP activity while Lipo246 cells exhibited the least, however, Lipo246 cells were the

only ones to display active MMP-2. The LACW shows a consistent decrease in MMP activity with inhibition of MDM2 by SAR. However, it is not an ideal candidate for a peptide substrate for our controlled drug delivery system because it did not show preferential degradation at higher levels of MDM2 amplification in hydrogel encapsulation experiments which measure net proteolytic activity.

Figure 18 of the QGIW peptide shows cleavage by active MMP-1 by Lisa-2, Lipo863, and Lipo246 cell lines without SAR, and Lisa-2 and Lipo863 with SAR. It shows cleavage by pro MMP-2 by all cell lines with and without SAR. It shows cleavage by active MMP-2 by Lipo863 and Lipo246 with and without SAR. Finally, it shows cleavage by pro MMP-9 (molecular weight = 92 kDa) by all cell lines with and without SAR. Upon SAR treatment, active MMP-1 activity decreased for Lisa-2 cells by 72%, for Lipo863 cells by 60% ($p<0.05$), and for Lipo246 cells by 100% as shown by the decrease in fluorescence intensity in Figure 20. Upon SAR treatment, pro MMP-2 activity decreased for Lipo246 cells by 48% and increased by 4% for Lisa-2 cells and by 43% for Lipo863 cells as shown in Figure 20. Upon SAR treatment, active MMP-2 activity decreased for Lipo246 cells by 86% and increased by 31% for Lipo863 cells. Upon SAR treatment, pro MMP-9 activity increased for Lisa-2 cells by 96%, for Lipo863 cells by 21%, and for Lipo246 cells by 59% as shown by the increase in fluorescence intensity in Figure 20. The increased expression levels of MMP-1 by Lipo863 cells seen in the PCR experiments correspond to evident active MMP-1 activity seen in the zymography gels and the increased degradation of the QGIW peptide seen in the hydrogel encapsulations. Based on the zymographic analysis of the QGIW peptide, MMP-1 and MMP-2 are the primary MMPs secreted by LPS cells that are

responsible for its degradation. The selectivity of this peptide could be improved by making it more specific to these two MMPs.

Figure 19 of the RSLS peptide shows cleavage by active MMP-1 by Lipo863 with and without SAR. It also shows cleavage by active MMP-2 by Lisa-2, Lipo863, and Lipo246 without SAR treatment, and Lipo246 with SAR treatment. Finally, it shows cleavage by pro MMP-9 by Lisa-2, Lipo863, and Lipo246 without SAR treatment and Lisa-2 and Lipo246 with SAR treatment. Upon SAR treatment, active MMP-1 activity decreased for Lipo863 cells by 38% as shown by the decrease in fluorescence intensity in Figure 20. Upon SAR treatment, active MMP-2 activity decreased for Lisa-2 cells by 100%, for Lipo863 cells by 100%, and for Lipo246 cells by 36% as shown by the decrease in fluorescence intensity in Figure 20. Upon SAR treatment, pro MMP-9 activity decreased for Lipo863 cells by 100% and increased by 47% for Lisa-2 cells and by 46% for Lipo246 cells as shown by Figure 20.

Minimal MMP activity in PLGL and LACW zymography gels correspond to minimal peptide degradation seen in the hydrogel encapsulations. Higher levels of MMP activity in QGIW and RSLS indicate potential peptide sequences for incorporation into controlled drug delivery system.

It is important to note the limitations of peptide zymography experiments. The technique is used to determine which MMPs might be contributing to peptide degradation. However, electrophoresis results in separation of the MMP-TIMP complexes and sometimes results in

incomplete re-folding of the enzymes. Therefore, MMPs which may otherwise be inactive appear active and vice versa. Therefore, the peptide zymography needs to be coupled with hydrogel encapsulation, which is a better measure of net proteolytic activity. Based on these results, QGIW and RSLS are the leading candidates for an MMP-sensitive drug delivery system targeting LPS cell lines with elevated *Mdm2*. Future work will focus on improving the selectivity of these peptides towards degradation by the specific MMPs secreted by LPS cells (MMP-1 and -2).

Conclusion and Future Direction

DDLPS is a unique type of cancer that is highly, locally recurrent with a poor patient prognosis. DDLPS intervention strategies are a critical area of research due to the need to gain a better understanding of disease progression and for the development of new, effective treatment modalities. In this study, we investigated how to create a targeted drug delivery system to treat DDLPS based on preferential degradation of a drug-containing substrate by cells with high expression levels of *Mdm2*.

In the results for the PCR experiments, we observed a trend where the cell lines with overamplification of *Mdm2* (Lipo863 and Lipo246), showed significant increases in expression of MMP-1, MMP-2, and MMP-14 compared to the cell line with no overamplification of *Mdm2*, Lisa-2. When treated with MDM2 inhibitor, SAR, evident decreases in MMP expression was observed in both Lipo863 and Lipo246 cell lines. Lisa-2 cells, on the contrary, showed a general increase in MMP expression upon SAR treatment, likely due to the fact that they otherwise display no amplification of *Mdm2*.

Based on the results of the qRT-PCR, we designed peptide substrates that were preferentially degraded by these MMPs. We observed a trend of preferential degradation according to *Mdm2* overamplification status in both the QGIW and RSLS peptide substrates. Despite the fact that the differences in peptide degradation were not significant, both peptides present viable options for substrates in an LPS-targeted drug delivery system.

Fluorescent peptide zymography was used to determine which MMPs were responsible for peptide degradation. We observed cleavage by active MMP-1, pro and active MMP-2, and pro MMP-9. MMP activity levels were highest in the QGIW and RSLS peptides, which aligned with the results of the hydrogel encapsulation experiments. Both peptides showed preference for degradation by MMP-1 and MMP-2.

Future studies should focus on building upon our work by refining the peptide substrate to be incorporated into the targeted drug delivery system. QGIW and RSLS peptides are optimal candidates for this process and can be adjusted to further cater to the MMPs actively contributing to peptide degradation (MMP-1 and MMP-2). The next step would then involve actually designing the targeted drug delivery system and integrating the ideal, modified peptide substrate. Ultimately, the goal of this research is to improve therapeutic methods to treat and save patients suffering from DDLPS.

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